FEBS Letters 582 (2008) 2542–2548

Morphogenesis of human endothelial cells is inhibited by DAB2 via Src

Maurizio Orlandini[a,1], Sara Nucciotti[a,1], Federico Galvagni[a], Monia Bardelli[a], Marina Rocchigiani[a], Felice Petraglia[b], Salvatore Oliviero[a,*]

[a] *Dipartimento di Biologia Molecolare, Università degli Studi di Siena, via Fiorentina, 1-53100 Siena, Italy*
[b] *Dipartimento di Pediatria, Ostetricia e Medicina della Riproduzione, Università degli Studi di Siena, Policlinico Le Scotte, 53100 Siena, Italy*

Received 19 March 2008; revised 29 May 2008; accepted 13 June 2008

Available online 26 June 2008

Edited by Michael R. Bubb

Abstract Disabled-2 (DAB2) is an adaptor protein implicated in signal transduction pathways and in protein traffic regulation. Here, we show that DAB2 is highly expressed in human endothelial cells. DAB2 silencing in endothelial cells by lentiviral-mediated small hairpin RNA expression affects cell migration and differentiation into capillary-like structures while increasing cell proliferation and viability. DAB2 knockdown causes activation of the Src-FAK signal pathway, extracellular-signal regulated kinase and c-Jun NH_2-terminal kinase activation, and inhibition of p38 phosphorylation. In DAB2 silenced endothelial cells, pharmacological inhibition of Src with its specific inhibitor PP2 abolishes focal adhesion kinase activation and restores differentiation of endothelial cells. These results suggest that DAB2, via Src and focal adhesion signaling, plays a role in human endothelial cell function.

Keywords: Angiogenesis; Disabled-2; RNA interference; Focal adhesion kinase; Src; Mitogen-activated protein kinase

1. Introduction

Dab2 (also known as *p96* and *DOC-2*) is an adaptor protein implicated in growth factor signaling [1,2], endocytosis [3], cell adhesive function [4,5], and hematopoietic cell differentiation [6]. Like other adaptor proteins, Dab2 contains protein-binding domains and phosphorylation sites, and lacks catalytic domains [7]. Dab2 contains an amino-terminal phosphotyrosine-binding (PTB) domain and a carboxy-terminal proline-rich domain (PRD), which have been shown to associate with the low-density lipoprotein receptor [3,8], myosin VI [9], the integrin β subunits [10–12], TAK1 [5], Grb2 [1,2], c-Src [13], Smad2/3 [14], DIP1/2 [15], Dvl-3 [16], and axin [17]. These interactions have been shown to modulate protein trafficking, cytoskeleton organization, cell adhesion and migration, and cell signaling of various receptor protein–tyrosine kinases. Dab2 thus plays a pivotal role in the control of cellular homeostasis.

Dab2 knock-out mice are embryonic lethal for defective visceral endodermal cell organization [18], and Dab2 regulates both lipoprotein trafficking [19] and generation and maintenance of epithelial polarity [20]. Although there is no evidence that Dab2 plays a role in angiogenesis in the mouse [18,19] or humans [21], in zebrafish it has been shown that *Dab2* expression is restricted to the presumptive endothelial cells of the forming vasculature of the trunk [22] and in Xenopus early development *Dab2* is expressed in the blood vessels and regulates angiogenesis acting as a specific mediator of activin-like signaling pathway for VEGF induction [23].

In this report, we show that DAB2 is expressed in mouse endothelium and in human umbilical vein endothelial cells (HUVEC), suggesting that DAB2 plays a role in mammalian angiogenesis. As endothelial cells play a pivotal role during angiogenesis and function as both transducer and effectors of local environmental signals [24], we investigated the function of DAB2 in human endothelial cells. In HUVEC, DAB2 silencing impaired migration and endothelial cell differentiation into capillary-like structures. Moreover, DAB2 silencing led to the activation of Src-FAK signaling and the modulation of mitogen-activated protein kinase (MAPK) phosphorylation. Together our data demonstrate a role of DAB2 in the control of endothelial cell function by modulating Src activity.

2. Materials and methods

2.1. Immunofluorescence microscopy

For histological analysis, SWISS mice were killed and portions of tail were dissected and frozen in liquid nitrogen. Umbilical cords collected from uncomplicated pregnancies were dissected and frozen in liquid nitrogen. Cryostat sections (20 μm) were cut and fixed in 3% paraformaldehyde for 20 min at room temperature. HUVEC were isolated from umbilical cords and cultured in M199 culture medium containing 20% fetal bovine serum and growth supplements. Cells were seeded on glass coverslips and fixed in 3% paraformaldehyde. Specimens were treated as previously described [25]. The primary antibodies used were: rabbit anti-von Willebrand factor (Dako), rat anti-CD31 (PharMingen), mouse anti-Dab2/p96 (Transduction Laboratories), rabbit anti-phospho-Y925 FAK, rabbit anti-Src (32G6), and rabbit anti-phospho-Y416 Src (Cell Signaling). Fluorescent images were captured using a Leica TCS SP2 laser scanning confocal microscope.

2.2. DAB2 RNA interference

Small hairpin (shRNA) cassette was cloned, and the recombinant lentiviruses were produced as previously described [25]. Briefly, oligonucleotides coding for human DAB2 (GeneBank accession number NM_001343) and unrelated (synthetic construct, GeneBank accession

*Corresponding author. Fax: +39 0577 234903.
E-mail address: oliviero@unisi.it (S. Oliviero).

[1]These authors contributed equally to the work.

Abbreviations: DAB2, Disabled-2; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; FAK, focal adhesion kinase; JNK, c-Jun NH_2-terminal kinase

0014-5793/$34.00
doi:10.1016/j.febslet.2008.06.025

number DQ092361) shRNA were designed to contain a sense strand 5′-GTCCATACAGAATGGCGTAAA-3′ (DAB2, clone 22), 5′-GTATCTGAAAGAGAACAGAAC-3′ (DAB2, clone 47), and 5′-GCCACAAGTTCAGCGTGTC-3′ (unrelated) followed by a spacer, their reverse complementary strand, and an RNA polymerase III transcriptional stop signal. The complementary oligonucleotides were phosphorylated, annealed, and cloned into the lentiviral vector. HEK293 cells were transiently transfected using Lipofectamine™ 2000 reagent (Invitrogen) following the manufacturer's instructions. Recombinant lentiviruses were harvested, concentrated by ultracentrifugation, and used for infection experiments. Vector infectivity was evaluated titrating shRNA-expressing virus by quantitative RT-PCR of a common lentiviral genome region when compared with the unrelated shRNA vector.

2.3. Cell viability, proliferation, and adhesion assays

The effect of DAB2 silencing on HUVEC viability was evaluated by spectrophotometric measurement of the mitochondrial dehydrogenase activity, apoptosis was assessed by annexin V/propidium iodide double-staining assay, and cell proliferation was evaluated by thymidine incorporation as described in Supplementary methods online.

Adhesion assay was performed in plates coated with 1% gelatin in PBS. Infected HUVEC were grown in complete medium, trypsinized, plated into coated wells (2×10^4 cell/well), and incubated at 37 °C (5% CO_2). At different time points cells were washed and the number of adherent cells was determined by MTT assay using linear curves plotted on known amounts of cells.

2.4. Tube formation and migration assays

Formation of capillary-like tube structures was examined on Matrigel (growth factor reduced, BD Biosciences). Matrigel (0.1 ml) was polymerized on 24-well plates and 2×10^4 cells were seeded in complete growth medium. The Src family tyrosine kinase inhibitor PP2 was purchased from Calbiochem. For PP2 treatment, cells were grown for 6 h in complete medium in the presence of 2 μM PP2 and then seeded in Matrigel containing 2 μM PP2. After 20 h, images of tube formation were captured using an inverted microscope (Leica DMIL) equipped with a Nikon DXM1200 digital camera. Chemotaxis analysis was performed as previously described with Boyden transwell chambers [26].

2.5. Immunoprecipitation and immunoblotting analyses

Immunoprecipitation and immunoblotting analyses were performed as previously described [25]. Cultures were starved in serum-free M199 medium containing 0.25% bovine serum albumin for 16–20 h. Cell lysates were immunoprecipitated with anti-Src (32G6), anti-Src-agarose (clone GD11, Upstate), or anti-phospho-Y925 FAK antibodies. To improve the detection of immunoprecipitated proteins we used ExactaCruz™ reagents (Santa Cruz Biotechnology). In immunoblotting the following primary antibodies were used: anti-Src (32G6); anti-p38; anti-phospho-p38 (T180/Y182); anti-p44/42 MAPK; anti-phospho-p44/42 MAPK (T202/Y204); anti-SAPK/JNK; anti-phospho-SAPK/JNK (T183/Y185) (Cell Signaling); anti-β-tubulin (D10), anti-FAK (Santa Cruz Biotechnology); anti-β-actin (Sigma–Aldrich).

3. Results

3.1. DAB2 is expressed in vascular tissues

To investigate DAB2 expression in mammalian endothelium, we first performed on mouse tail double immunohistochemical staining for Dab2 and CD31, a marker of endothelial cells. The analysis of vessel sections revealed overlapping of Dab2 and CD31 signals, demonstrating that Dab2 is expressed in mouse endothelial tissues (Fig. 1A). To determine whether DAB2 is expressed on human endothelium, we analyzed cross-sections and primary endothelial cells from the umbilical vein by immunostaining. The analyses by indirect immunofluorescence revealed that in the umbilical vein endothelium DAB2 co-localizes with the von Willebrand factor, a marker of endothelial cells (Fig. 1B), and in HUVEC endogenous DAB2 was localized in numerous discrete spots as previously described for other cell types (Fig. 1C) [3,8].

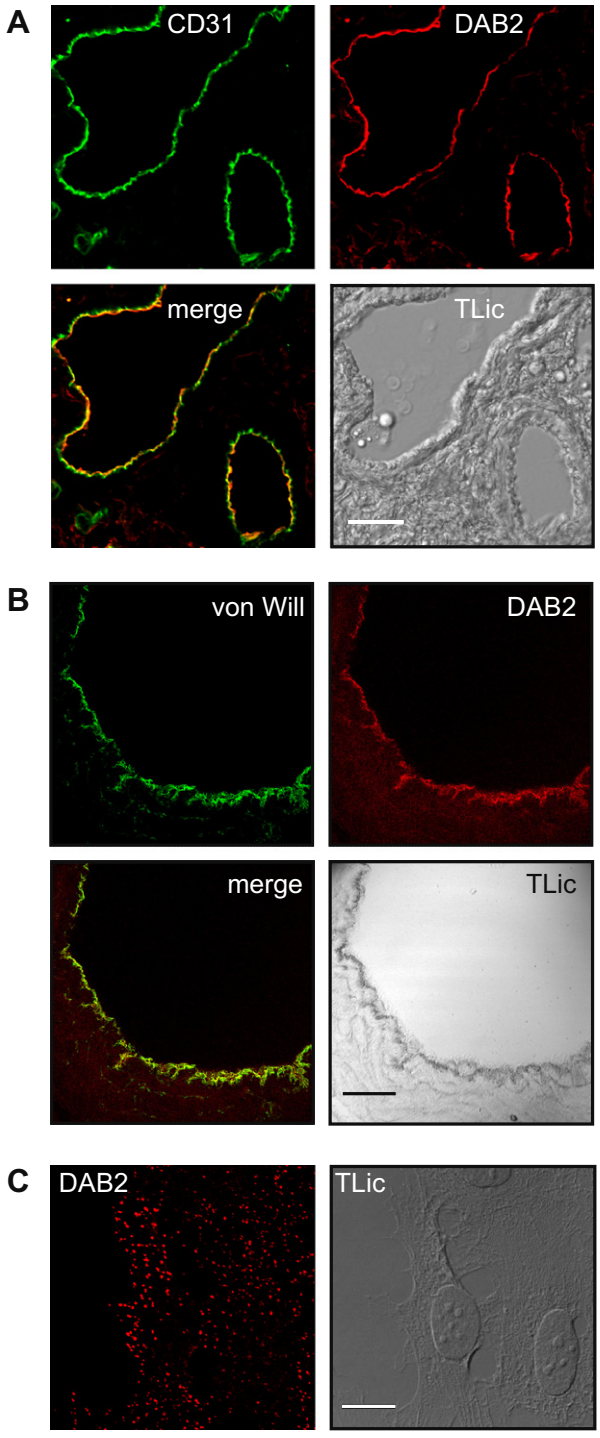

Fig. 1. DAB2 localization in endothelial tissues. (A) Section of endothelial vessels through mouse tail stained by immunofluorescence using anti-CD31 and anti-DAB2 antibodies. Scale bar, 45 μm. (B) Vein section through human umbilical cord stained by immunofluorescence using anti-von Willebrand factor (von Will) and anti-DAB2 antibodies. Scale bar, 150 μm. The merge of the double staining (merge) and the transmitted light interference contrast (TLic) of the same cross-sections are shown. (C) HUVEC were grown in complete medium and analyzed by immunofluorescence using anti-DAB2 antibodies. TLic, transmitted light interference contrast. Scale bar, 11 μm.

3.2. DAB2 knockdown inhibits cell migration and in vitro angiogenesis

To elucidate the role of DAB2 in human endothelial cells we adopted the RNAi technology approach. Western blot analysis of DAB2 in HUVEC showed that this protein is constitutively expressed in these cells and its level is not modulated by starvation or VEGF treatment (Fig. 2A). To silence DAB2 we generated two lentiviral constructs (clones 22 and 47) expressing two independent DAB2 shRNAs. HUVEC infected with lentiviruses expressing either DAB2 shRNA showed a reduced DAB2 protein expression while no DAB2 reduction was observed upon infection with a lentivirus expressing an unrelated shRNA (Fig. 2B). In the adult, endothelial cells are normally quiescent, but in response to appropriate stimuli (injury and disease), they become activated and are co-ordinately involved in the process of proliferation, migration, and differentiation. In order to determine the effect of DAB2 silencing on angiogenesis, we investigated the ability of HUVEC to form capillary-like structures when cultured on Matrigel, which is a process mimicking sprouting and tube formation during angiogenesis in vivo. Transduction of HUVEC before suspension in Matrigel with lentiviruses carrying the control shRNA did not affected tubulogenesis (Fig. 2C). If, however, cells were infected with lentiviruses carrying DAB2 shRNA, tubulogenesis was strongly impaired, since DAB2 silenced cells produced few and broken tubes unable to connect to each other. As both migration and adhesion are critical for angiogenesis, we performed in vitro migration and adhesion assays to investigate the role of DAB2 in these processes. Analysis of cell migration using the Boyden chamber assay showed a significant reduction of migration of DAB2 knockdown endothelial cells compared with control cells (Fig. 2D). Similar results were obtained through wound closure assays (data not shown). Cell attachment assays were performed on culture plates coated with gelatin. HUVEC silenced for DAB2 showed a significant increased adhesion to the matrix compared to cells expressing the control shRNA (Fig. 2E). Taken together these results suggest that DAB2 plays a function in endothelial cell adhesion

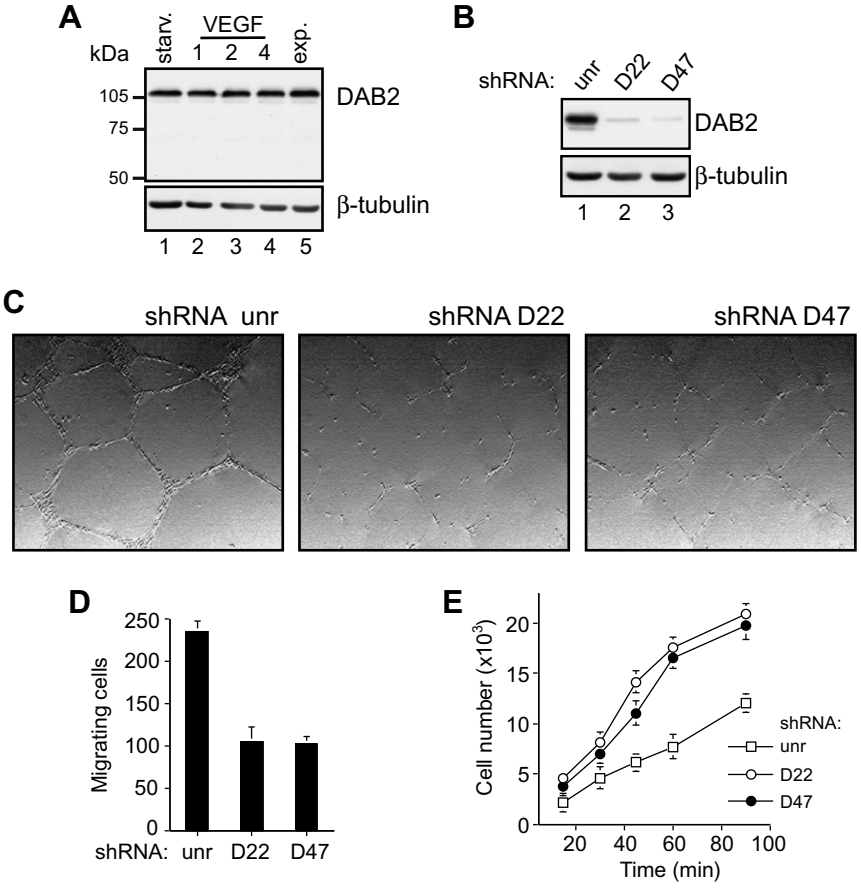

Fig. 2. DAB2 silencing affects in vitro adhesion, migration, and tube formation in human endothelial cells. (A) HUVEC were cultured in complete medium (exp.) or serum-starved for 16 h (starv.) and treated for 1, 2 and 4 h with 10 ng/mL VEGF (VEGF) purchased from Sigma. Cell extracts were analyzed by Western blotting using anti-DAB2 and anti-β-tubulin antibodies to confirm equal loading. (B) HUVEC were infected with a lentiviral vector expressing unrelated (unr) or DAB2 shRNA clone 22 (D22) and clone 47 (D47). Cell extracts from shRNA expressing HUVEC were analyzed by Western blotting using anti-DAB2 and anti-β-tubulin antibodies. (C) Infected HUVEC were seeded on Matrigel and the formation of capillary-like structures was observed 20 h after seeding. Multiple experiments indicated that both the length of the tubules and the number of branch points were reduced in DAB2 silenced cells (D22 and D47) respect to the control (unr). A representative experiment is shown (×40 magnification). (D) Infected endothelial cells were grown in growth factor-depleted culture medium for 20 h and plated in the upper compartment of Boyden chambers. Migratory cells were stained and counted under a light microscope. (E) In adhesion assay, infected HUVEC were seeded into 24-well plates coated with gelatin. At the indicated times, only adherent cells were revealed by MTT assay. The OD values were related to cell number by linear curves plotted on known amounts of cells. Results were expressed as means ± S.E. of at least four independent experiments each in triplicate.

and migration, which affects the organization of endothelial cells into vessel structures.

3.3. In human endothelial cells, DAB2 silencing affects Src-FAK signaling

A possible candidate for DAB2-dependent activity is Src, since Src is a tyrosine kinase located at focal adhesions involved in cell motility, adhesion, proliferation, and survival. In tumour cells DAB2 is known to be a negative regulator of Src activation via binding to its SH3 domain [13,27]. Accordingly, we found that in HUVEC DAB2 co-immunoprecipitated with Src (Fig. 3A). To investigate the effect of DAB2 knockdown on endogenous unstimulated Src activity in endothelial cells, we performed immunoprecipitation analyses from serum-depleted cells. As shown in Fig. 3B, Src phosphorylation at Y416, a protein modification that is closely correlated with the active form of Src kinase, was significantly increased in DAB2 silenced cells respect to control endothelial cells. Moreover, immunofluorescence analyses showed that Src phosphorylation at Y416 was associated with peripheral adhesions and it was strongly increased in DAB2 silenced cells compared to DAB2 expressing cells (Fig. 3C). Emerging evidence supports the important role for FAK in the endothelial cell motility response [28]. Since in cancer cells specific Src-dependent phosphorylation of FAK Y925 is associated with the ability of cells to dynamically regulate cell adhesions [29], we next investigated the phosphorylation state of FAK in endothelial cells silenced for DAB2. We performed immunoprecipitation analyses of cell extracts from quiescent cells by using anti-Y925 FAK antibodies. As shown in Fig. 4A (lanes 1–3), DAB2 knockdown determined a significant increase of FAK phosphorylation at Y925 respect to control endothelial cells. Double-staining with both anti-Y925 FAK and anti-DAB2 antibodies revealed that FAK phosphorylation on Y925 was localized at the tips of cellular protrusions and it was strongly increased in DAB2

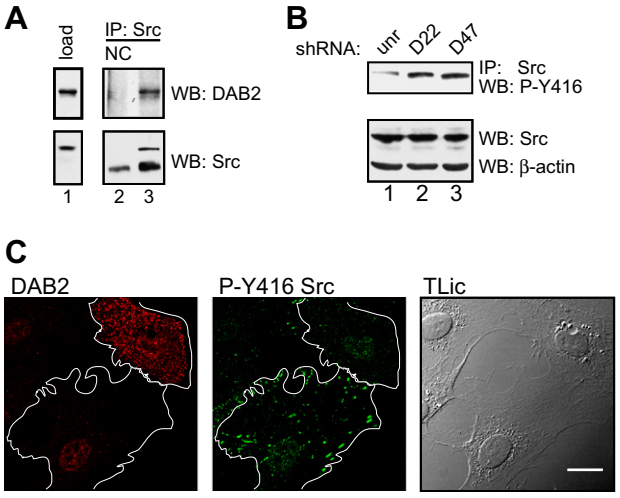

Fig. 3. DAB2 knockdown affects c-Src activation in human endothelial cells. (A) Cell extracts from serum-induced endothelial cells were immunoprecipitated with anti-Src antibodies. The immunoprecipitation was analyzed by Western blotting with anti-DAB2 and anti-Src antibodies. As control a non-correlated antibody was used (NC). To confirm equal loading, whole cell lysates (load) were analyzed by Western blotting with anti-DAB2 and anti-Src antibodies. (B) HUVEC were infected with a lentiviral vector expressing unrelated (unr) or DAB2 shRNA clone 22 (D22) and clone 47 (D47). Cell extracts from serum-starved cells were immunoprecipitated with anti-Src antibodies. The immunoprecipitation was analyzed by Western blotting with anti-phospho-Y416 Src antibodies (P-Y416). To confirm equal loading, whole cell lysates were analyzed by Western blotting with anti-Src and anti-β-actin antibodies. (C) Infected HUVEC as in B were mixed and plated onto the same glass coverslip to obtain microscope fields containing DAB2 expressing and not-expressing cells. Cells were grown in serum-deprived medium for 16 h and analyzed by immunofluorescence using anti-DAB2 (DAB2) and anti-phospho-Y416 Src (P-Y416 Src) antibodies. A representative microscope field is shown and a white line indicates cell boundary. TLic, transmitted light interference contrast of stained cells. Scale bar, 16 μm.

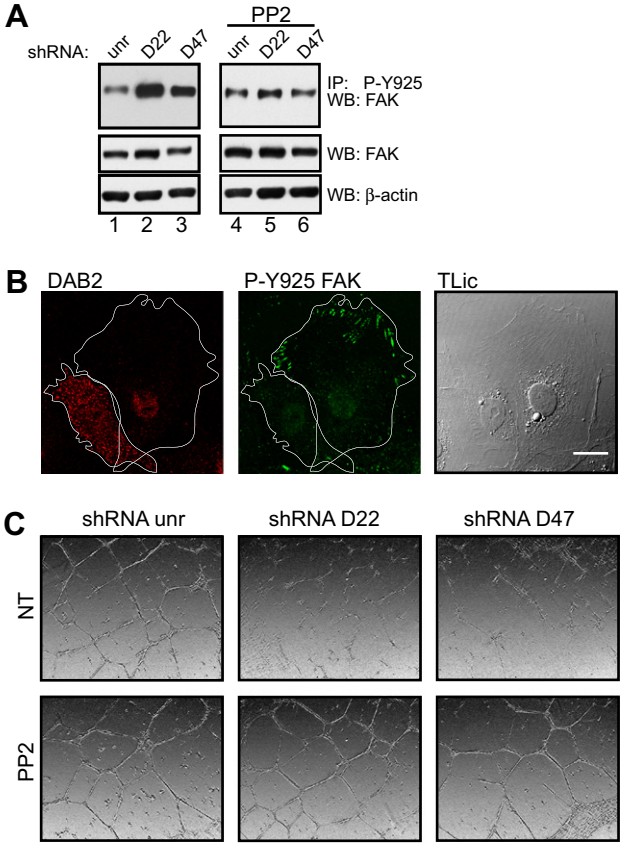

Fig. 4. FAK phosphorylation at Y925 by means of activated c-Src affects tube formation in endothelial cells. HUVEC were infected with a lentiviral vector expressing unrelated (unr) or DAB2 shRNA clone 22 (D22) and clone 47 (D47). (A) Infected HUVEC were serum-starved for 16 h with or without the presence of 2 μM Src kinase inhibitor PP2. Cell extracts were immunoprecipitated using anti-phospho-Y925 FAK (P-Y925) antibodies and the immunoprecipitation was analyzed by Western blotting with anti-FAK antibodies (FAK). To confirm equal loading, whole cell lysates were analyzed by Western blotting with anti-FAK and anti-β-actin antibodies. (B) Infected HUVEC were mixed and plated on glass coverslips to obtain in the same microscope field cells expressing or not DAB2 protein. Cells were grown in serum-deprived medium and analyzed by immunofluorescence using anti-DAB2 (DAB2) and anti-phospho-Y925 FAK antibodies (P-Y925 FAK). A representative microscope field is shown and a white line indicates the cell border. Tlic, transmitted light interference contrast of stained cells. Scale bar, 18 μm. (C) Infected HUVEC were grown on Matrigel in the presence or not of 2 μM c-Src kinase inhibitor PP2 and the formation of capillary-like structures was observed 20 h after seeding. A representative experiment is shown (×40 magnification). Results were expressed as means ± S.E. of at least four independent experiments each in triplicate.

silenced cells compared to the control (Fig. 4B). To demonstrate the involvement of Src in DAB2-dependent activation of FAK tyrosine phosphorylation, quiescent HUVEC were cultured in the presence of 2 μM PP2, a selective Src inhibitor. Immunoprecipitation analysis showed that in DAB2 silenced endothelial the levels of Y925 FAK phosphorylation was decreased by PP2 treatment (Fig. 4A, lanes 4–6). Importantly, tubulogenesis assays demonstrated that in the presence of PP2 DAB2 silenced HUVEC were able to gather into clumps and form the intricate tubular networks seen with control cells. Of note, PP2 treatment per se did not alter the ability of control cells to form tubular structures. Thus, PP2 treatment restored in DAB2 silenced cells the ability to form capillary-like structures in vitro. The above results demonstrate that DAB2, controlling Src activation, regulates FAK, which is responsible of the adhesion properties and the in vitro angiogenic cellular responses of human endothelial cells.

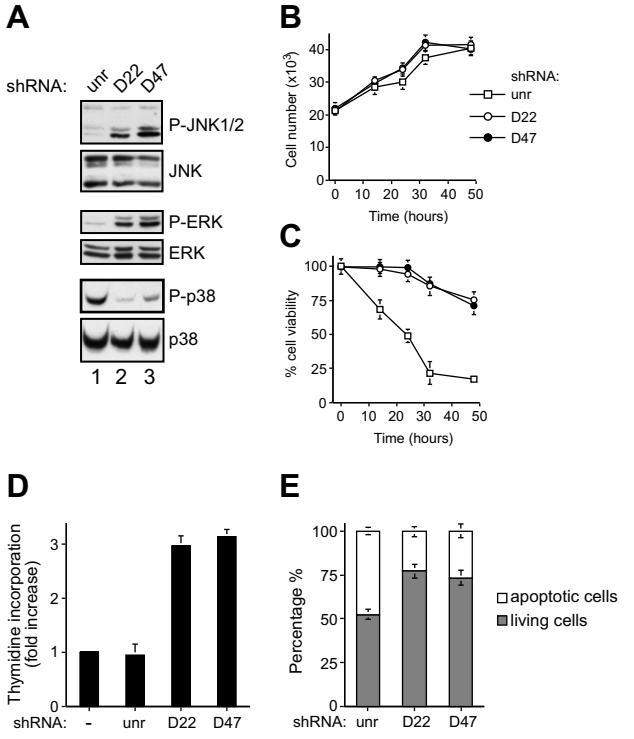

Fig. 5. Inhibition of DAB2 expression affects MAPK signal transduction and enhances cell survival and proliferation in human endothelial cells. HUVEC were infected with a lentiviral vector expressing unrelated (unr) or DAB2 shRNA clone 22 (D22) and clone 47 (D47). (A) To investigate the activation status of the MAPK family, infected cells were serum-starved for 18 h and cell extracts were analyzed by Western blotting using anti-phospho-MAPK family (P-MAPK) and anti-MAPK family (MAPK) antibodies to confirm equal loading. (B) Effect of DAB2 knockdown on number of HUVEC growing in complete culture medium. 2×10^4 cells were seeded and MTT assays were performed at the times indicated. To estimate cell number, the OD values of MTT were plotted on linear curves obtained from known amounts of cells (see Materials and Methods). (C) Effect of DAB2 silencing on HUVEC growing in serum-depleted medium (M199 culture medium containing 0.25% bovine serum albumin). At the times indicated, MTT assays were performed and the ratios of the absorbance of viable cells relative to initial seeded cells were expressed as cell viability percentage. (D) Cell proliferation expressed as thymidine uptake in infected or non-infected (−) HUVEC growing in growth factor-free culture medium for 36 h. (E) The effect of DAB2 silencing on survival of infected HUVEC growing in growth factor-depleted culture medium for 20 h. The apoptotic percentage is the sum of early, intermediate, and late apoptotic cells as shown in supplementary data (Fig. S1C). All presented data were expressed as means ± S.E. of 3–5 independent experiments, each in triplicate.

3.4. DAB2 affects MAPK signaling, endothelial cell viability and proliferation

Since Y925 in FAK is involved in intra- and inter-molecular signalling crosstalk and activation of downstream pathways [28], we next examined the impact of DAB2 knockdown on the activation status of the MAPK family, ERK-1/2, JNK and p38 serine/threonine protein kinases that are involved in proliferation and migration of endothelial cells. As expected, in growth factor-depleted conditions, control endothelial cells did not show ERK phosphorylation. Conversely, quiescent endothelial cells silenced for DAB2 showed a strong ERK-1/2 stimulation respect to control cells (Fig. 5A). ERK phosphorylation was sustained, because it could be revealed at different time points during starvation (data not shown). A similar profile was also observed for JNK phosphorylation while we observed a strong reduction of p38 phosphorylation in DAB2 silenced cells. These results suggested an involvement of DAB2 in endothelial cell viability and proliferation. Therefore, we investigated the effect of DAB2 silencing in HUVEC by proliferation and survival assays. The number of viable cells was estimated at different time points of culturing. Cells expressing DAB2 shRNA grown in complete growth medium did not show any variation in cell number respect to cells infected with control shRNA (Fig. 5B). However, when cells were grown in growth factor-depleted medium, we observed in control cells a strong decrease in cell viability, while in DAB2 silenced cells viability was reduced for only 25% even after 48 h of starvation (Fig. 5C). DNA synthesis, determined in growth factor-depleted cells by thymidine uptake, was increased in endothelial cells expressing DAB2 shRNA compared to control cells (Fig. 5D). Moreover, flow cytometric assays with annexin V/propidium iodide double staining showed that, in growth factor-depleted conditions, apoptosis was reduced in endothelial cells expressing DAB2 shRNA respect to the control (Fig. 5E). Taken together these results demonstrate that in human endothelial cells, DAB2 depletion affects MAPK family activity and this change has different effects on cell behavior.

4. Discussion

During angiogenesis endothelial cells, in response to growth factor stimulation, proliferate, migrate, and aggregate in a coordinated process involving formation and disassembly of cell adhesion sites to form vessels structures [24]. In this report we analyzed the function of the adaptor protein DAB2 in human endothelial cells. By immunofluorescence and Western blotting we showed that DAB2 is highly expressed in human endothelium and by RNA interference technology we observed that DAB2 down-regulation affects endothelial cell functions crucial to angiogenesis like cell proliferation, survival, migration, and the alignment of endothelial cells in capillary-like structures. These in vitro results contrast with *Dab2* knock out, which did not show vasculogenic defects. This discrepancy could be explained by gene compensations in the knock-out animals, as observed with other genes [30].

DAB2 is a putative tumor suppressor. It is downregulated in various types of tumor and is capable of strongly inhibiting cell growth and proliferation in many cell types [31–33]. In normal and malignant prostatic epithelial cells, DAB2 is a negative regulator of c-Src and the downstream Erk activation [13,27]. Accordingly, we observed that in endothelial cells DAB2 forms a complex with Src and that DAB2 depletion increased Src activity, which was associated with peripheral adhesions. At focal adhesions the integrin cytoplasmic domains bind to a protein complex including Src and FAK. These signaling molecules act to control focal contact turnover during cell motility, although the molecular mechanisms are only partly understood [34]. FAK has been shown to trigger several downstream signaling pathways which mediate different phenotypes including cell spreading, growth, and survival [34,35]. As FAK phosphorylation at Y925 is linked to adhesion turnover [29,36], we analyzed the activation of FAK in endothelial cells. We found that DAB2 knockdown induced Y925 FAK phosphorylation and that in DAB2 silenced HUVEC the ability to form capillary-like tube structures was restored after pharmacological inhibition of Src by PP2. These results suggest that DAB2 controls migration and capillary-like tube formation by modulating FAK activity via Src. Sustained activation of Src-FAK signaling, as a result of DAB2 depletion, might increase the maturation of focal complexes to stabilize focal adhesions or slowdown their turnover, resulting in inhibition of endothelial cell migration, which is a coordinated process involving formation and disassembly of cell adhesion sites.

We observed that in DAB2 silenced endothelial cells FAK activation correlates with ERK and JNK phosphorylation. These results are consistent with previous studies that Y925 FAK phosphorylation is important in promoting ERK and JNK activity [28,36]. The regulation of these signaling pathways explains the observed increases in cell proliferation and survival following DAB2 knockdown. Moreover, we found that DAB2 silenced endothelial cells showed a balance of MAPK activities, with inhibition of p38 activation concomitant with an increase of ERK phosphorylation. These results might explain the dual effect of DAB2 knockdown, which is responsible for the increased proliferation and survival together with inhibition of cell motility and tube formation. In conclusion, our results suggest that DAB2 exerts its function as a signaling mediator in the control of endothelial cell functions by modulating FAK and its downstream signaling.

Acknowledgements: This study was supported by grants from Associazione Italiana ricerca sul Cancro (AIRC) and Fondazione Monte dei Paschi di Siena (MPS).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.06.025.

References

[1] Xu, X.X., Yi, T., Tang, B. and Lambeth, J.D. (1998) Disabled-2 (Dab2) is an SH3 domain-binding partner of Grb2. Oncogene 16, 1561–1569.
[2] Zhou, J. and Hsieh, J.T. (2001) The inhibitory role of DOC-2/DAB2 in growth factor receptor-mediated signal cascade. J. Biol. Chem. 276, 27793–27798.
[3] Mishra, S.K., Keyel, P.A., Hawryluk, M.J., Agostinelli, N.R., Watkins, S.C. and Traub, L.M. (2002) Disabled-2 exhibits the properties of a cargo-selective endocytic clathrin adaptor. EMBO J. 21, 4915–4926.
[4] Rosenbauer, F., Kallies, A., Scheller, M., Knobeloch, K.P., Rock, C.O., Schwieger, M., Stocking, C. and Horak, I. (2002) Disabled-2 is transcriptionally regulated by ICSBP and augments macrophage spreading and adhesion. EMBO J. 21, 211–220.
[5] Hocevar, B.A., Prunier, C. and Howe, P.H. (2005) Disabled-2 (Dab2) mediates TGFβ-stimulated fibronectin synthesis through TAK1 and activation of the JNK pathway. J. Biol. Chem. 280, 25920–25927.
[6] Tseng, C.P., Huang, C.H., Tseng, C.C., Lin, M.H., Hsieh, J.T. and Tseng, C.H. (2001) Induction of disabled-2 gene during megakaryocyte differentiation of k562 cells. Biochem. Biophys. Res. Commun. 285, 129–135.
[7] Pawson, T. and Scott, J.D. (1997) Signaling through scaffold, anchoring, and adaptor proteins. Science 278, 2075–2080.
[8] Morris, S.M. and Cooper, J.A. (2001) Disabled-2 colocalizes with the LDLR in clathrin-coated pits and interacts with AP-2. Traffic 2, 111–123.
[9] Spudich, G., Chibalina, M.V., Au, J.S.-Y., Arden, S.D., Buss, F. and Kendrick-Jones, J. (2007) Myosin VI targeting to clathrin-coated structures and dimerization is mediated by binding to Disabled-2 and PtdIns(4,5)P2. Nat. Cell Biol. 9, 176–183.
[10] Calderwood, D.A., Fujioka, Y., de Pereda, J.M., Garcia-Alvarez, B., Nakamoto, T., Margolis, B., McGlade, C.J., Liddington, R.C. and Ginsberg, M.H. (2003) Integrin β cytoplasmic domain interactions with phosphotyrosine-binding domains: a structural prototype for diversity in integrin signaling. Proc. Natl. Acad. Sci. USA 100, 2272–2277.
[11] Huang, C.L., Cheng, J.C., Liao, C.H., Stern, A., Hsieh, J.T., Wang, C.H., Hsu, H.L. and Tseng, C.P. (2004) Disabled-2 is a negative regulator of integrin alpha(IIb)beta(3)-mediated fibrinogen adhesion and cell signaling. J. Biol. Chem. 279, 42279–42289.
[12] Prunier, C. and Howe, P.H. (2005) Disabled-2 (Dab2) is required for transforming growth factor β-induced epithelial to mesenchymal transition (EMT). J. Biol. Chem. 280, 17540–17548.
[13] Zhou, J., Scholes, J. and Hsieh, J.T. (2003) Characterization of a novel negative regulator (DOC-2/DAB2) of c-Src in normal prostatic epithelium and cancer. J. Biol. Chem. 278, 6936–6941.
[14] Hocevar, B.A., Smine, A., Xu, X.X. and Howe, P.H. (2001) The adaptor molecule Disabled-2 links the transforming growth factor β receptors to the Smad pathway. EMBO J. 20, 2789–2801.
[15] Wang, Z., Tseng, C.P., Pong, R.C., Chen, H., McConnell, J.D., Navone, N. and Hsieh, J.T. (2002) The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. J. Biol. Chem. 277, 12622–12631.
[16] Hocevar, B.A., Mou, F., Rennolds, J.L., Morris, S.M., Cooper, J.A. and Howe, P.H. (2003) Regulation of the Wnt signaling pathway by Dab2. EMBO J. 22, 3084–3094.
[17] Jiang, Y., Prunier, C. and Howe, P.H. (2008) The inhibitory effects of Dab2 on Wnt signaling are mediated through axin. Oncogene 27, 1865–1875.
[18] Yang, D.-H. et al. (2002) Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. Dev. Biol. 251, 27–44.
[19] Morris, S.M., Tallquist, M.D., Rock, C.O. and Cooper, J.A. (2002) Dual roles for the Dab2 adaptor protein in embryonic development and kidney transport. EMBO J. 21, 1555–1564.
[20] Yang, D.-H., Cai, K.Q., Roland, I.H., Smith, E.R. and Xu, X.-X. (2007) Disabled-2 is an epithelial surface positioning gene. J. Biol. Chem. 282, 13114–13122.
[21] Fazili, Z., Sun, W., Mittelstaedt, S., Cohen, C. and Xu, X.X. (1999) Disabled-2 inactivation is an early step in ovarian tumorigenicity. Oncogene 18, 3104–3113.
[22] Song, H.-D., Sun, X-J., Deng, M., Zhang, G.-W., Zhou, Y., Wu, X.-Y., Sheng, Y., Chen, Y., Ruan, Z., Jiang, C.-L., Fan, H.-Y., Zon, L.I., Kanki, J.P., Liu, T.X., Look, A.T. and Chen, Z. (2004)

Hematopoietic gene expression profile in zebrafish kidney marrow. Proc. Natl. Acad. Sci. USA 101, 16240–16245.
[23] Cheong, S.M., Choi, S.C. and Han, J.K. (2006) Xenopus Dab2 is required for embryonic angiogenesis. BMC Dev. Biol. 6, 63–76.
[24] Carmeliet, P. (2003) Angiogenesis in health and disease. Nat. Med. 9, 653–660.
[25] Orlandini, M., Spreafico, A., Bardelli, M., Rocchigiani, M., Salameh, A., Nucciotti, S., Capperucci, C., Frediani, B. and Oliviero, S. (2006) Vascular endothelial growth factor-D activates VEGFR-3 expressed in osteoblasts inducing their differentiation. J. Biol. Chem. 281, 17961–17967.
[26] Marconcini, L., Marchio, S., Morbidelli, L., Cartocci, E., Albini, A., Ziche, M., Bussolino, F. and Oliviero, S. (1999) c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. Proc. Natl. Acad. Sci. USA 96, 9671–9676.
[27] Zhoul, J., Hernandez, G., Tu, S.-W., Huang, C.-L., Tseng, C.-P. and Hsieh, J.-T. (2005) The role of DOC-2/DAB2 in modulating androgen receptor-mediated cell growth via the nongenomic c-Src-mediated pathway in normal prostatic epithelium and cancer. Cancer Res. 65, 9906–9913.
[28] Mitra, S.K. et al. (2006) Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. Oncogene 25, 5969–5984.
[29] Brunton, V.G., Avizienyte, E., Fincham, V.J., Serrels, B., Metcalf III, C.A., Sawyer, T.K. and Frame, M.C. (2005) Identification of Src-specific phosphorylation site on FAK: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. Cancer Res. 65, 1335–1342.
[30] Sage, J., Miller, A.L., Perez-Mancera, P.A., Wysocki, J.M. and Jacks, T. (2003) Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature 424, 223–228.
[31] Mok, S.C., Chan, W.Y., Wong, K.K., Cheung, K.K., Lau, C.C., Ng, S.W., Baldini, A., Colitti, C.V., Rock, C.O. and Berkowitz, R.S. (1998) DOC-2, a candidate tumor suppressor gene in human epithelial ovarian cancer. Oncogene 16, 2381–2387.
[32] Tseng, C.P., Ely, B.D., Pong, R.C., Wang, Z., Zhou, J. and Hsieh, J.T. (1999) The role of DOC-2/DAB2 protein phosphorylation in the inhibition of AP-1 activity. An underlying mechanism of its tumor-suppressive function in prostate cancer. J. Biol. Chem. 274, 31981–31986.
[33] Wang, S.C., Makino, K., Xia, W., Kim, J.S., Im, S.A., Peng, H., Mok, S.C., Singletary, S.E. and Hung, M.C. (2001) DOC-2/hDab-2 inhibits ILK activity and induces anoikis in breast cancer cells through an Akt-independent pathway. Oncogene 20, 6960–6964.
[34] Mitra, S.K., Hanson, D.A. and Schlaepfer, D.D. (2005) Focal adhesion kinase: in command and control of cell motility. Nat. Rev. Mol. Cell Biol. 6, 56–68.
[35] Shen, T.-L. et al. (2005) Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J. Cell Biol. 169, 941–952.
[36] Grijelmo, C., Rodrigue, C., Svrcek, M., Bruyneel, E., Hendrix, A., de Wever, O. and Gespach, C. (2007) Proinvasive activity of BMP-7 through SMAD4/src-independent and ERK/Rac/JNK-dependent signaling pathways in colon cancer cells. Cell. Signal. 19, 1722–1732.